Using RNAi in *C. elegans* to Demonstrate Gene Knockdown Phenotypes in the Undergraduate Biology Lab Setting

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Abstract: RNA interference (RNAi) is a powerful technology used to knock down genes in basic research and medicine. In 2006 RNAi technology using *Caenorhabditis elegans* (*C. elegans*) was awarded the Nobel Prize in medicine and thus students graduating in the biological sciences should have experience with this technology. However, students struggle conceptually with the molecular biology behind the RNAi technology and find the technology difficult to grasp. To this end, we have provided a simple, streamlined and inexpensive RNAi procedure using *C. elegans* that can be adopted in upper level biology classes. By using an unknown RNAi-producing bacteria, students perform novel techniques, observe and determine which mystery gene was knocked down based on phenotype and experience a new research organism. By bringing this technology to the undergraduate lab bench, the gap between blackboard concept and proof of concept can be bridged.

Keywords: *C. elegans*, RNAi, gene knockdown

INTRODUCTION

During their tenure in the undergraduate science environment, science students should become familiar with experimental techniques and skills needed for their postgraduate careers. A frequent challenge for professors in the biological sciences is finding effective ways to ensure student engagement and provide students with the opportunity to experience original and valuable research techniques (Adams, 2009). The goal of any instructor should be to introduce new techniques and model organisms that relate to the real world science environment as well as enhance student engagement.

In 2006, the Nobel Prize in Medicine was awarded jointly to Andrew Z. Fire and Craig C. Mello for their discovery of RNA interference (RNAi), a gene silencing mechanism by double-stranded RNA. The number of scientific publications involving RNAi has jumped from zero in 1998 to over in 4500 in 2005. Currently there are over 13,200 publications referencing RNAi in the literature, reflecting the explosion of RNAi research and the importance of the technology. In 2002, *Science* Magazine named RNAi "technology of the year" and in 2003 *Forbes Magazine* called RNAi "Biotech's billion dollar breakthrough" (Adams, 2004).

RNAi technology spans both basic scientific research and medicine and is an important concept and technology that graduating students in the biological sciences should understand. For example, RNAi therapies have been used in the treatment of ocular diseases like macular degeneration, viral infections including hepatitis and HIV, cancers, inflammatory conditions and neurodegenerative diseases (Lares et al. 2010). However, the concept of how a small RNA molecule can elicit a radical gene knockdown phenotype is often difficult for students to conceptually understand.

Caenorhabditis elegans is a very popular model organism utilized in many fields of study. *C. elegans* is a simple organism amenable to studies in genetics and development, cell biology, neuroscience, evolution and ecology (Girard et al. 2007). The popularity of *C. elegans* rises from its genetic manipulability, fully described developmental life-cycle, fully sequenced genome, ease of maintenance, short and prolific life cycle and small body size (Leung et al., 2008)

This laboratory exercise provides a hands-on approach to demonstrating the Nobel Prize winning RNAi mechanism and introduces students to an important and popular research model, *C. elegans*. By bringing this technology to the undergraduate lab bench, we can bridge the gap between blackboard concept and proof of practice. Because RNAi is multi-disciplinary and crosses many scientific fields, this exercise is applicable to the undergraduate developmental biology, cell and molecular biology or biotechnology lab setting.

MATERIALS AND METHODS

Acquiring *C. elegans* strains

Wild-type *C. elegans* strains are available free of charge to educational institutions through the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, St.Paul, by emailing a request describing the requested strain and a brief statement of intended use. Requests can be emailed to cgc@umn.edu. Strains of their choice are sent on NGM petri plates seeded with *E. coli* OP50 as feeding bacteria.

Preparing *E. coli* OP50 food source

C. elegans utilizes *E. coli* OP50 as a food source when the *E. coli* is spread as a lawn on culture plates. A starter *E. coli* OP50 culture can also be obtained through CGC. A starter culture is prepared by aseptically transferring a single colony from the streak plate into 250ml sterile Luria Broth (10g Bacto-tryptone, 5 g Bacto-yeast, 5g NaCl, H_20 to 1-liter, pH to 7.0 using 1M NaOH). The innoculated cultures grow overnight at 37°C after which the bacteria can be used to seed NGM plates. The liquid culture is stored at 4°C and is stable for several months (Stiernagle, 2006).

Preparing NGM petri plates

Standard Falcon 60mm petri plates are used to maintain *C. elegans*. Nematode Growth Medium (NGM) agar is prepared by mixing 3g NaCl, 17g agar, 2.5g peptone and 975ml H_20 in a 2-liter flask which is autoclaved for 50 minutes. After the flask cools to 55°C in a water bath, 1ml 1M $CaCl_2$, 1ml 5mg/ml cholesterol dissolved in ethanol, 1ml 1M $MgS0_4$ and 25ml 1M KPO_4 are added and mixed. Using sterile technique, warm NGM mixture is poured into the 60mm petri plates until they are 2/3 full. The plates sit for 2-3 days at room temperature before they are seeded to allow moisture to evaporate and to detect contamination (Stiernagle, 2006).

Seeding NGM Plates

A volume of 50 µl of OP50 bacterial culture is aseptically transferred to the center of an NMG plate on a flat surface. The bacterial lawn will grow overnight at room temperature. The seeded plates are stored on a countertop in a sealed air-tight container for 2-3 weeks (Stiernagle, 2006).

Maintaining Worm Cultures

The worms are maintained by transferring a chunk of NGM agar every three days to a new seeded bacterial plate. This is performed using a sterilized spatula to cut a 0.5cm x 0.5cm piece of agar with worms from an old plate and flipping the chunk over and placing it near the bacterial lawn of a fresh seeded plate. The worms will crawl out from under the chunk and feed on the new lawn (Stiernagle, 2006). Worms are best maintained at 20°C in a humidified incubator, but development can be accelerated at 25°C. Cross usage of the incubator with other lab species is acceptable. Old plates should be autoclaved to kill any biohazardous material prior to disposal.

Preparing RNAi bacteria

RNAi bacterial libraries can be purchased at Source BioScience LifeSciences ($15,500) or from Thermo Scientific **(***C. elegans* ORF-RNAi library Comprehensive coverage for RNAi screening). However, purchasing libraries can be costly for a small undergraduate lab budget. Although buying a whole library (19,762 clones) provides the ability to knock down any gene, this is not necessary for the confines of this undergraduate lab experiment which only requires five to ten strains. Contacting *C. elegans* labs and asking if they are willing to send a few RNAi bacterial strains is less costly. Most labs are willing to donate a few RNAi bacterial clones for undergraduate student use. The RNAi bacterial cultures are prepared in 5ml LB with 50µg/ml ampicillin or 10µg/ml tetracycline and 25µg/ml carbenicillin depending on the antibiotic resistance genes present in the plasmid (plasmid maps are provided by the donating lab). The cultures are grown overnight in a shaking incubator at 37°C.

Preparing RNAi/NGM plates

Standard NGM plates are prepared as described above with the addition of 25µg/ml carbenicillin and 1mM IPTG to the NGM agar mix prior to pouring plates. The plates must be poured 4 days before being seeding with RNAi bacteria to allow time for them to dry, but covers should remain on during this time. If the plates are wet, the RNAi phenotype will not be as strong (Stiernagle, 2006). Due to addition of IPTG, plates must be stored in the dark. The plates must be stored at 4°C. After the plates have dried, 50µl of overnight RNAi bacterial culture is spread and left to dry for 2-3 days in the dark at room temperature.

Preparing *C. elegans* transfer tool

The transfer tools are prepared using Fisher brand 5 ¾ inch glass pipets. Platinum wire (99.95%), 0.05% iridium, 0.01-inch diameter, 30G can be purchased at Tritech Research (PT-9901, www.TritechResearch.com). The transfer tools are prepared by cutting a 1-inch piece of platinum wire and flattening one end with standard 5-inch flat-nose pliers. The wire is bent into an S-shape and the non-flattened end is placed into the open end of the glass pipet. The wire is set into place by melting the glass under a flame.

Worms are transferred using bacteria as a sticky source. The bacteria stick to the flat surface of the transfer tool when it is touched to the seeded lawn. The worms are "lifted" as they stick to the OP50 bacteria and deposited to a new OP50 lawn for

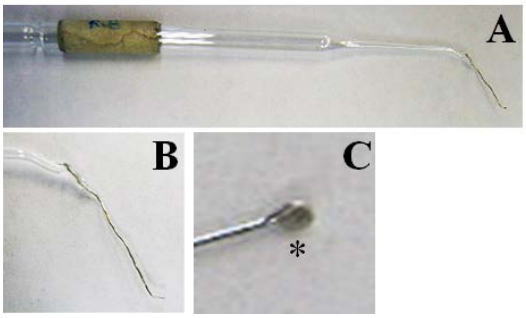


Fig.1. A. Full length glass picking tool. B. Close up view of platinum wire inserted and melted into glass pipet tip. C. Close up view of flattened end used for worm lifting. Asterisks show lifting surface in side or front views

continual culture. The wire tool should be flamed between transfers. Transfers are performed under a dissection microscope.

Microscopy

The specimens are observed under a Leica Zoom 2000 stereomicroscope with 10x ocular eye pieces and a zoom magnification range from 10.5x to 45x with both reflected and transmitted light illumination.

Transferring worms to RNAi plate

Using the transfer tool two or three L2 to L3 stage worms are moved to an unseeded NGM plate and given 30 minutes to allow the worms to wiggle off excess OP50 bacteria. *C. elegans* prefer to eat OP50 bacteria to RNAi bacteria and excess OP50 will cause a weaker RNAi phenotype. Using the transfer tool, the RNAi bacteria are scooped up from an RNAi seeded plate. Using the transfer tool with the RNAi bacteria stuck to it, the worms are picked up from the unseeded plate. The worms are transferred onto the RNAi seeded plate. The plates should be kept in the dark on a countertop. The phenotypes can be scored 3 days later. The worms are staged using a standard staging reference (http://www.wormatlas.org/).

Student Exercise

The exercise began with student groups growing a control and an unknown RNAi bacterial culture and treating the L2 to L3 staged *C. elegans* worms. The project was intended to supplement a lecture on the molecular mechanisms of RNAi, provide a hands-on activity using RNAi and *C. elegans* and demonstrate the technology. The goal of the lab was for student groups to determine what gene the unknown RNAi knocked down based on the phenotype of their progeny.

Students first observed wild-type worms using a stereomicroscope. These observations introduced the students to the basic worm morphology, life stages of the worms, use of the stereomicroscope and the transfer tool. After students became competent, they were able to determine which worms were suitable to pick for RNAi treatments (L2 to L3 as referenced in the standard staging series). Students fed L2 to L3 staged worms with the unknown RNAi-producing bacteria as well as with a control (empty vector) bacteria. After 3 days students scored the phenotype of the progeny.

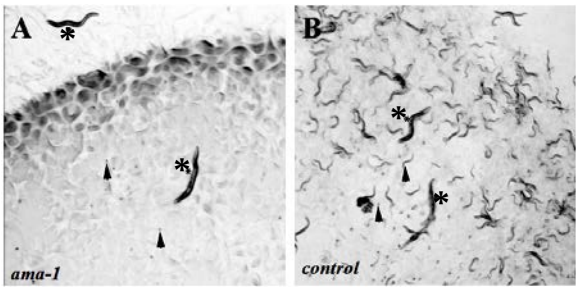


Fig.2. A. RNAi knockdown of *ama-1* gene resulting in dead eggs due to knockdown of RNA polymerase II. Dead eggs are noted with arrow. Asterisks denote original parent worms. B. RNAi knockdown using control empty vector. Live eggs produced by parental worms (shown by asterisks) grow and multiply. Arrows show live eggs and larva

Student Assignment: Lab Day 1

1. Draw and anatomically describe the wild-type worms on your petri dish. Note and describe the differences among all worm stages from gastrula, L1, L2, L3, L4, young adult and adult. Select three worms at the L2 or L3 stage and transfer them to an unseeded plate. What were the key anatomical structures that defined the L2 or L3 stage worm?

2. Transfer the worms from the unseeded plate to the RNAi plate and also to a control plate. Why must an unseeded plate be used? What are you trying to avoid in your transfer process and why? How would it affect your results?

Student Assignment: Lab Day 2 (3 days post-treatment)

1. Obtain your RNAi treated and control worms. Describe what you see. How many worms are now on the plates?

2. Describe and draw any phenotypic changes you see in the progeny. Count the number of progeny that display a phenotype and rank the phenotypes along a scale from 0 to 3; 3 demonstrating a strong phenotype and 0

Table 1. Genes knocked down using RNAi technology.

Gene	Normal Function	Knockdown Phenotype	Reference
bli-4	Post-embryonic cuticle development and stability	Fluid filled blisters	Page, 2007
unc-22	Regulates actomyosin contraction-relaxation cycles	Uncoordinated head muscle twitching	Edgley, 2006
dpy-5	Cuticle procollagen necessary for body length	Abnormal cuticle, shortened football shaped body	Page, 2007
lon-2	Negative regulator of growth factor signaling/regulates body length	Elongation of body length	Page, 2007
ama-1	Encodes large subunit of RNA polymerase II required for mrna transcription	The laid eggs never hatch	Blackwell, 2006
rol-6	Cuticle collagen necessary for cuticle morphology	Rolls around, horseshoe shaped	Page, 2007

demonstrating no phenotype (a wild type worm). You will define intermittent phenotypes graded 1 and 2 from your observations. From these numbers, calculate the total number of progeny that display a phenotype. Why do you think some worms do not demonstrate a knockdown phenotype and appear wild-type? Why do some show a stronger phenotype than others?

3. Monitor the worms' behavior, but do not touch them. Do you see any behavioral differences in your RNAi treated worms? Touch your worms gently with your tool. Describe their behavior. Are they different than control worms and, if so, how?

4. Based on your progeny phenotype, speculate on what gene/pathway you might have knocked down with your RNAi treatments and explain why.

5. Based on your progeny phenotype, log on to www.wormbase.org and determine which gene you knocked down. Were you surprised? Did this fit with your speculated pathway above?

RESULTS

Genes that were knocked down in this experiment included *bli-4*, *unc-22*, *rol-6*, *lon-2*, *dpy-5* and *ama-1*. Table 1 lists the gene wild-type product and description of the knockdown phenotype. As an example, Figure 2 demonstrates representative phenotypes for the RNAi knockdown of *ama-1* from student treatments. Adult worms shown by asterisk are approximately 1mm in length. Students were given a list of possible loci their RNAi might target. From the observed phenotypes, students had to determine which mystery gene they knocked down.

Laboratory Evaluation

A pre-exercise survey/quiz determined that only 20% of 15 students had heard of RNAi, and only 13% knew what RNAi could be used for. Sixty seven percent of students had heard of *C. elegans*, but only 27% knew why it was used as a research model (Figure 3).

Additionally, student enjoyment of the project was surveyed. Sixty seven percent of students reported they extremely enjoyed working on a technology that won the Nobel Prize and 33% reported they strongly enjoyed it.

DISCUSSION

For many students, the mechanism of RNAi may seem overwhelmingly complex and confusing initially. While explaining a methodology in a classroom is an essential first start, laboratory exercises provide a stronger and hands-on method for teaching methodology and concept. Many undergraduate biology majors will be pursuing careers in research, academia or medicine and thus introducing RNAi at the college level is essential. Furthermore, introducing a novel organism like *C. elegans* at the undergraduate level further engages students in scientific inquiry.

Our data demonstrate that junior and senior level biology students had very little familiarity with the *C. elegans* model or the RNAi technology at the beginning of the project, despite its relevance in the real world science environment. This demonstrates the need for such an experiment in the undergraduate setting. When students considered how well they liked working on this project, 67% of students reported that they extremely enjoyed working on this project and 33% reported they enjoyed this project a great deal. Students were very excited and even shocked to see the phenotypes that they created! Seeing how knocking down one gene could lead to such dramatic phenotypes was exciting and emphasized the process they learned about in lecture. Although explaining and drawing out the molecular mechanisms of RNAi in a lecture setting is important for students, performing the experiment and generating a mutant phenotype provides them hands-on proof. Having a tangible product of a molecular mechanism occurring is a helpful educational tool. Additionally, having student groups use different examples of RNAi enhanced the exercise as students were able to see other group's phenotypes. At the end of the project, each student group presented their data in oral presentation format to the class in a mock scientific conference format. Student enjoyment is an

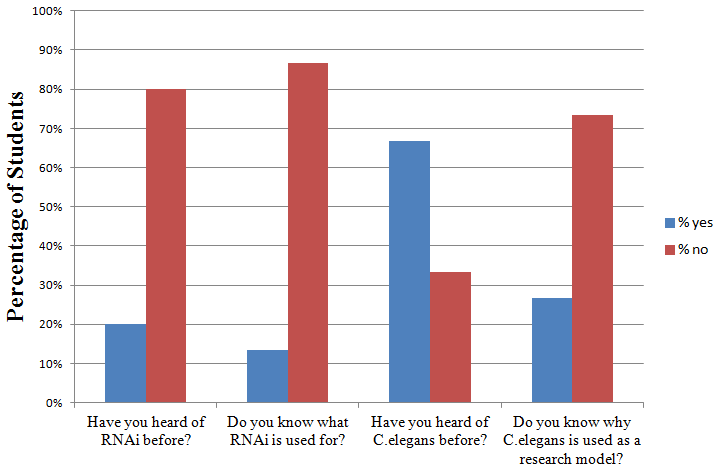


Fig. 3. Pre-exercise survey determining student familiarity with RNAi.

important factor when designing or planning lab exercises. If the exercise does not stimulate student interest or spark curiosity, then students are not invested in the project and will get less out of it (Adams, 2009).

Although the lab was successful, students did encounter problems with the laboratory exercise. Initially, transferring worms from one plate to another was a challenge. If the transfer tool does not have a well-flattened surface, the bacteria and worms tend not to stick well. Additionally, students had difficulty with microscope depth of field and struggled to either find the worms for lifting or place them gently on the transfer plate. Students tended to gouge the agar plate with their tool, creating holes where the worms would nest. Furthermore, the RNAi bacteria are not as "sticky" as OP50 bacteria. Thus, several practice rounds of worm transfers were necessary before performing the RNAi exercise and students were encouraged to make multiple transfer tools. Furthermore, the students' first round of phenotypes were rather weak due to residual OP50 bacteria that were transferred. The worms prefer the OP50 bacteria over the RNAi-producing bacteria and as such did not eat the RNAi bacteria resulting in a weaker phenotype. For the second round of treatments, an additional transfer to another unseeded plate was incorporated to allow the worms to wiggle off excess OP50.

Demonstrated here is a simple RNAi exercise using the *C. elegans* model organism amenable to the undergraduate lab setting. The experiment can be performed in one week or two, depending on how many replicates the instructor wishes to perform and can easily fit into a tightly scheduled undergraduate lab calendar. In this exercise, students learned about *C. elegans* as a research model organism, the life cycle of the worms and the practicality of using worms in research. Furthermore, the lab takes a complex molecular mechanism like RNAi and demonstrates proof of concept. Students enjoyed the hands-on approach of working with *C. elegans* and were excited about the mutant phenotypes they generated utilizing RNAi technology.

Lastly, the lab module could be followed up with a truly investigative open-ended research project. For example, students could select pairs of genes to knock down. Of particular interest could be selection of genes which are described to have no individual knock down phenotype, for example *sin-3* with *tbx-34* or *set-31* with *scrm-6*. Perhaps the dual gene knock down would yield an interesting result. These experiments would bridge the gap from introductory "skill building" lab activities to truly investigative scientific research.

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